

Applicants: David M. Stern et al.
U.S. Serial No.: 09/638,647
Filing Date: August 14, 2000
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Please amend the subject application as follows:

In the specification:

Please amend the specification under the provisions of 37 C.F.R. §1.121(c) as follows. A marked-up version of the amended specification wherein the deleted material is in brackets and the inserted material is underlined is attached hereto as **Exhibit A**.

Please delete the paragraphs beginning on page 4, line 14 and ending on page 4, line 28 and insert the following paragraphs:

a¹ -- **Figures 4A, 4B, 4C and 4D.** ABAD expression in Tg PD-ABAD mice (+) compared with nontransgenic littermate controls (-). Figure 4A (Northern) and Figure 4B (Western) analysis of homogenates of cerebral cortex. Equal amounts of RNA (note approximately equal intensity of 28S ribosomal RNA band on the ethidium bromide stained gel) and protein were loaded in each lane. Figures 4C-4D show immunohistochemical identification of ABAD in cerebral cortex from a Tg PD-ABAD mouse (Figure 4C) and a nontransgenic littermate control (Figure 4D).

Figure 5. ABAD expression in brain subregions of Tg PD-ABAD mice compared with nontransgenic littermate controls (nonTg). Immunoblotting was performed protein extracts of brain homogenates derived from the indicated brain subregion. --

Please delete the paragraph beginning on page 5, line 25 and ending on page 6, line 6 and insert the following paragraph:

a² -- **Figures 7A-7D.** Induction of stroke in Tg PD-ABAD mice. Figures 7A-7B. Tg PD-ABAD mice and nonTg littermates were subjected to middle cerebral artery occlusion and were evaluated 24 hrs after the ischemic insult to determine neurologic deficit score (Figure 7B), and, following

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sacrifice, infarct volume (Figure 7A). Figures 7C-7D. At the same time point, cerebral cortex was harvested to determine ATP, lactate and β -hydroxybutyrate (BHB) levels determined on extracts of whole brains (from animals subjected to the stroke procedure 24 hrs previously) from Tg PD-ABAD or nonTg control mice (N=5, in each case). Data is reported as the mean \pm SD ($P < 0.04$ for ATP and $P < 0.03$ for lactate). Transient middle cerebral artery occlusion model of stroke in mice: comparison of infarct volume in Tg PD-ABAD and nontransgenic littermate controls (nonTg). * $P < 0.05$. --

Please delete the paragraphs beginning on page 6, line 27 and ending on page 7, line 11 and insert the following paragraphs:

-- **Figures 11A-11B.** Semiquantitative analysis of synaptophysin immunoreactivity in hippocampus of Tg PD-ABAD/hAPP, Tg PD-ABAD, Tg hAPP, and nontransgenic littermate control mice at 4 months of age.

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Figures 12A1, 12A2, 12A3, 12A4, and 12B. Increased expression of activated caspase-3 in cerebral cortex from Tg PD-ABAD/hAPP mice. Figures 12A1-12A4, immunostaining for activated caspase-3. Figure 12B, quantitation of immunocytochemical results from multiple fields of all mice in each of the experimental groups. Scale bar, 10 μ m.

Figures 13A-13B. Northern analysis (Figure 13A) and immunoblotting (Figure 13B) of E16 cortical neuron cultures with 32 P-labelled human ABAD cDNA (Figure 13A) or anti-human ABAD IgG (Figure 13B). (+) indicates neurons obtained from Tg PD-ABAD mice and (-) indicates neurons are from nontransgenic littermate controls. --

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Please delete the paragraph beginning on page 14, line 7 and insert the following paragraph:

ad -- The transgenic non-human mammals of the present invention will provide insights with respect to how and where protein interactions occur in Alzheimer's Disease and thus provide more useful models for testing the efficacy of certain drugs in preventing or reducing the onset or progression of this disease. The transgenic non-human mammals of the present invention include recombinant genetic material comprised of a nucleic acid sequence encoding ABAD fused to specific promoters capable of expressing the protein in specific tissues such as nerve tissues generally and/or specific types of nerve tissue, e.g., the brain. --

Please delete the paragraph beginning on page 32, line 9 and insert the following paragraph:

ca -- Western blotting was performed on protein extracts of brain subregions. Proteins were extracted from minced pieces of brains by exposing the tissue to lysis buffer (Tris/HCl, 20 mM; pH 7.4; Triton X-100, 1%; phenylmethylsulfonylfluoride, 2 mM; EDTA, 1 mM, aprotonin, 10 μ g/ml; leupeptin, 10 μ g/ml) using a ratio of 1 ml of buffer per 0.5 gm of tissue. Extracts were then boiled in reducing SDS-sample buffer, and applied to SDS-PAGE (12%) according to Laemmli¹¹. Antibody to recombinant human ABAD was employed as described². --

Please delete the paragraph beginning on page 32, line 29 and ending on page 33, line 10 and insert the following paragraph:

ca -- Cross-breeding of Tg PD-[RAGE] ABAD mice with Tg hAPP mice. Tg mice overexpressing an alternatively spliced hAPP minigene that encodes hAPP695, hAPP751, and hAPP770 bearing mutations linked to familial AD (V717F, K670M/N671L) have been produced by Dr. Hsia¹⁴, and provided to us for use in cross-breeding

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studies with Tg PD-ABAD mice. In these mice, expression of the transgene is also driven by the PDGF B-chain promoter. Cross-breeding was performed and double-transgenic mice expressing both hAPP and PD-ABAD transgenes were identified with specific primers. The primers for the hAPP transgene were: 5'-GACAAGTATCTCGAGACACCTGGGGATGAG-3' (SEQ ID NO:6) and 3'-AAAGAACTTGTAGGTTGGATTTTCGTACC-5' (SEQ ID NO:7). PCR conditions for the amplifying the hAPP transgene were the same as those described above, and the size of the amplicon was 1169 bp. --

Please delete the paragraph beginning on page 39, line 15 and ending on page 40, line 2 and insert the following paragraph:

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-- Induction of stroke in Tg PD-ABAD mice. Functional consequences of overexpression of ABAD were first assessed in response to ischemic stress, the transient middle cerebral artery occlusion model. *Murine stroke model* Mice (C57BL6/J, male) were subjected to stroke according to previously published procedures³². Following anesthesia, the carotid artery was accessed using the operative approach previously described in detail³³, including division/coagulation of the occipital and pterygopalatine arteries to obtain improved visualization and vascular access. A nylon suture was then introduced into the common carotid artery, and threaded up the internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). Nylon (polyamide) suture material was obtained from United States Surgical Corporation (Norwalk, CT), and consisted of 5.0 nylon/13 mm length for 27-36 g mice, and 6.0 nylon/12 mm length for 22-26 g mice. After 45 minutes of occlusion, the suture was withdrawn to achieve a reperfused model of stroke. Although no vessels were tied off after the suture was removed, the external carotid arterial stump was cauterized to prevent frank hemorrhage. --

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Please delete the paragraph beginning on page 46, line 4 and insert the following paragraph:

-- Characterization of neurons isolated from Tg PD-ABAD mice.

Neuronal cultures were made from the cerebral cortex from E16 mouse embryos. These cultures were >90% neurons based on staining with anti-neurofilament antibody (not shown). Cultured neurons displayed high levels of ABAD expression based on Northern analysis and immunoblotting (Fig. 13). --

Please delete the paragraph beginning on page 47, line 10 and insert the following paragraph:

-- In each case, the *in vitro* and *in vivo* systems based on Tg PD-ABAD mice or cells derived from them are ideal for studying ABAD inhibitors, as well as for dissecting contributions of ABAD to physiologic/pathophysiologic outcomes. --

Please delete the paragraphs beginning on page 61, line 20 and ending on page 69, line 4 and insert the following paragraphs:

-- ABAD metabolism of β -hydroxybutyrate. The broad enzymatic properties of ABAD as an oxidoreductase suggested that it might facilitate cellular utilization of ketone bodies, such as D- β -hydroxybutyrate, a major energetic substrate during nutritional deprivation *in vivo*. First, we tested DL- β -hydroxybutyryl-CoA, a known substrate of bovine liver-derived hydroxyacyl-CoA type II (HADH II)/ABAD (17), with our purified *E. Coli*-derived human recombinant ABAD; the reaction fit Michaelis-Menton kinetics with K_m was 134 μ M and V_{max} 26.3 μ mol/min/mg. The latter result, obtained with a racemic DL mixture of β -hydroxybutyryl CoA, was similar to that observed previously with the bovine liver HADH II (17). Using the same preparation of recombinant ABAD, we then studied D- β -hydroxybutyrate; the reaction also fit best to Michaelis-Menton kinetics with K_m was 3.7 mM, and V_{max} was 4

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nmol/min/mg. With the L-form of β -hydroxybutyrate, K_m was 1.6 mM, and V_{max} 3.5 nmol/min/mg. In a purified system, ABAD is clearly more effective with β -hydroxybutyryl-CoA as a substrate (presumably this is the L-form, which is an intermediate in the fatty acid β -oxidation pathway in mitochondria). However, depending on physiological conditions, certain substrates may turn out to be more abundant, such as D- β -hydroxybutyrate during periods of starvation when levels of ketone bodies are elevated and, thus, could become relevant. In fact, plasma levels of β -hydroxybutyrate are reported to reach the millimolar range in animals and humans subject to nutritional deprivation (21-23). Furthermore, β -hydroxybutyryl CoA generated by acyl CoA dehydrogenase is another likely substrate of ABAD in a cellular milieu rich in β -hydroxybutyrate. Thus, ABAD would appear to have the potential to be pivotal for enhancing metabolism of β -hydroxybutyrate.

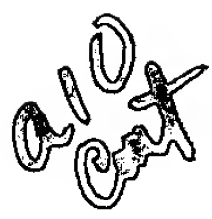
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Characterization of COS cells stably-transfected to overexpress ABAD. COS cells provided a useful model to test our concept that ABAD modulated the cellular response to nutritional stress because of their low endogenous expression of ABAD; low levels of mRNA were present and no antigen was detectable in lysates of wild-type COS cells. Following stable transfection with either pcDNA3 alone (vector), pcDNA3/wtABAD (encoding wild-type ABAD) or pcDNA3/mutABAD (encoding a mutant form of ABAD devoid of enzymatic activity; 14), cells were plated at limiting dilution and clones were prepared. Three types of clones were established, those expressing vector alone (COS/vector), wild-type ABAD (COS/wtABAD) and mutant ABAD (COS/mutABAD). Studies were performed with three representative clones of each type of stably-transfected COS cell. Whereas COS/vector cells displayed low levels of ABAD transcripts and antigen, comparable to control COS cells, COS/wtABAD cells showed high levels of ABAD mRNA and antigen. Subcellular fractionation studies on COS/wtABAD cells demonstrated the presence of ABAD

both in fractions 1-2 enriched for the endoplasmic reticulum marker GRP78 and in the mitochondrial pellet (fraction 6) containing cytochrome c. Similar experiments performed with COS/mutABAD stable transfectants displayed high levels of ABAD transcripts and antigen in a distribution analogous to that seen in COS/wtABAD cells. These data indicated that in COS/ABAD stable transfectants, the enzyme is present at the same sites previously observed in cells endogenously expressing ABAD or those transiently transfected to overexpress ABAD (13,14,16,19).

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ABAD and the response of COS cells to nutritional stress. In view of ABAD metabolism of D- β -hydroxybutyrate, we tested whether ABAD-transfected COS cells displayed enhanced ability to sustain nutritional stress in an environment where ketone bodies provided the principal energetic source. When COS/vector cells were placed in medium devoid of glucose and supplemented only with dialyzed serum and β -hydroxybutyrate, cellular functions became compromised. In the presence of D- β -hydroxybutyrate (10 mM), reduction of MTT was suppressed by days 4-5 and cellular energy charge decreased in parallel. Phase contrast microscopy showed COS/vector cells, initially with a spread morphology on the growth substrate, to become rounded up and toxic in appearance after four days under these conditions. Similar results were obtained when COS/vector cells were replaced with wild-type COS cells, and the same studies described above were performed (not shown). In contrast, COS/wtABAD cells better maintained MTT reduction and cellular energy charge in the presence of β -hydroxybutyrate. These changes in cellular properties were paralleled by maintenance of the morphologic phenotype of COS/wtABAD cells, compared with COS/vector transfectants in the presence of β -hydroxybutyrate. The effect of β -hydroxybutyrate to maintain cellular functions in COS/wtABAD cells was dose-dependent, as shown using the MTT reduction assay, and reached a plateau by 10 mM. Furthermore, experiments with lower concentrations of β -hydroxybutyrate (2.5 mM) displayed less effective

maintenance of cellular properties with COS/wtABAD cells (not shown). The requirement for enzymatically intact ABAD (wtABAD) for enhanced survival of COS/wtABAD cells in the presence of β -hydroxybutyrate was shown by experiments performed with COS/mutABAD cells. Though the crippled enzyme (mutABAD) was expressed at similar levels and with a similar subcellular distribution as the wild-type enzyme (the latter in COS/wtABAD cells), COS/mutABAD cells responded to glucose replacement with β -hydroxybutyrate as did COS/vector cells; there was a steady decline in MTT reduction and cellular energy charge.

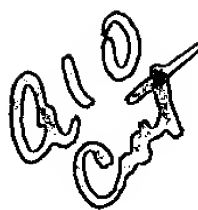
 NMR studies were performed on cells incubated with [^{13}C]-D- β -hydroxybutyrate (labelled in the C-2 and C-4 positions) to determine the effect of ABAD on the metabolism of the COS/wtABAD transfectants in medium devoid of glucose. [^{13}C]-labelled β -hydroxybutyrate enters the TCA cycle as [^{13}C -2]-labelled acetyl-CoA and is metabolized to α -ketoglutarate. Thus, C-4 gets labelled in the first turn of the TCA cycle, and, subsequent, labelling occurs in the C-3 and C-2 positions. As α -ketoglutarate is in rapid equilibrium with glutamate, the labelling of [^{13}C]-glutamate in the C-4, 3 and 2 positions was observed. Since the flux of [^{13}C]labelled acetyl-CoA via the TCA cycle is orientation conserved, the labelling of [^{13}C]-glutamate is greater in the C-4, compared with the C-3 and C-2, positions. Thus, labelling was evaluated in the C-4 position of glutamate in COS/wtABAD versus COS/vector in cell lysates and supernatants. In culture supernatants, NMR data demonstrated greater labelling of the C-4 resonances in glutamate in COS/wtABAD cells, 2-fold, compared with COS/vector cells ($p < 0.03$ at days 4, 6 and 8). Glutamine was not detected in supernatants from COS/wtABAD or COS/vector cells. The ^1H NMR analysis of these supernatants revealed that the fractional enrichment at glutamate C-4 was $58 \pm 3\%$ in COS/wtABAD transfectants compared with $41 \pm 2\%$ in COS/vector cells. These data are indicative of increased metabolism of exogenous [^{13}C]-labelled β -hydroxybutyrate in COS cells overexpressing ABAD. In contrast, there were no observed

differences between fractional enrichment at glutamate C-4 in cell lysates between COS/wtABAD and COS/vector cells, probably because glutamate is rapidly extruded into the medium (glutamine and glutamate levels in cell lysates were the same, comparing COS/wtABAD and COS/vector cells). The ^{13}C and ^1H NMR data demonstrate increased exogenous β -hydroxybutyrate utilization in the COS cells overexpressing wtABAD.

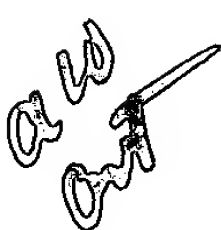
Upregulation of ABAD in response to cerebral ischemia. A severe form of metabolic stress is imposed by cerebral ischemia. Wild-type C57BL6 mice subjected to transient middle cerebral artery occlusion displayed increased levels of ABAD in neurons near the infarcted area, especially those in the penumbra, compared with the nonischemic hemisphere, using polyclonal antibody to recombinant mouse ABAD. Image analysis of multiple fields from sections similar to those shown in panels A-B demonstrated an 5-fold increase of ABAD antigen in cortical neurons consequent to stroke.

Characterization of Tg PD-ABAD mice. These data with wild-type mice subjected to cerebral ischemia suggested that upregulation of ABAD might be a component of the response to ischemic stress, and led us to make transgenic mice in which ABAD was overexpressed in cortical neurons. Three independent founders of Tg mice in which human ABAD is expressed under control of the human PDGF B-chain promoter have been identified and used to establish transgenic lines (at present backcrossed eight times into the C57BL6 background). Representative mice from each of these transgenic lines showed high levels of transgene activity at both the mRNA and protein levels in cerebral cortex. Immunoblotting performed on brain subregions from one line of Tg PD-ABAD mice, using an anti-human ABAD peptide antibody which selectively recognizes the human form of the protein, showed increased antigen especially in cerebral cortex and hippocampus, with a smaller increase in cerebellum. Immunohistochemical staining of ABAD in cerebral cortex confirmed high levels of antigen expression

in cortical neurons compared with nontransgenic littermates. Semiquantitative analysis of immunohistochemical results using antibody reactive with murine and human ABAD antigen (i.e., total ABAD antigen) indicated that there was an 3.5-4-fold increase in total ABAD antigen in cerebral cortex comparing Tg mice with nonTg littermate control mice. Induction of stroke in transgenic mice further elevated ABAD antigen another two-fold compared with nonTg controls (24 hrs after the ischemic episode; not shown). Growth (height/weight) and reproductive fitness (number and size of litters) was similar between Tg PD-ABAD mice and nontransgenic (nonTg) controls, and there were no overt neurologic symptoms or other phenotype evident in these mice noted to date.

 NMR analysis of ^{13}C - β -hydroxybutyrate metabolism in Tg PD-ABAD mice. Tg PD-ABAD and control mice were infused with D-[2,4- ^{13}C]-3-hydroxybutyrate. ^{13}C NMR spectra of cerebral cortical extracts from Tg PD-ABAD and nonTg littermate control (the latter spectra are not shown) mice illustrate labelling of glutamate and glutamine in the C-4, C-3 and C-2 positions, as well as GABA in the C-2 position, consistent with entry of [^{13}C]- β -hydroxybutyrate via 2-[^{13}C]-acetyl-CoA into the TCA cycle. The intensity of glutamate and glutamine C-4 resonance was 50% and 20% greater, respectively, in Tg PD-ABAD mice compared with nonTg littermates. The glutamate to glutamine ratio, based on C-4 resonance area, was 3.6 ± 0.3 in nonTg versus 2.1 ± 0.4 in Tg PD-ABAD mouse brains ($P < 0.03$). These data suggest that glutamine synthesis is more efficient in Tg PD-ABAD mouse brain compared with controls. The area of the ^{13}C -labelled C-2 resonance of GABA was also greater in Tg PD-ABAD mice (4.8 ± 0.3) than in nonTg controls (2.2 ± 0.5 ; $p < 0.04$). Such increased labelling of GABA is consistent with enhanced conversion of labelled glutamate to GABA in brains of Tg mice. ^1H NMR analysis of these extracts revealed that the fractional enrichment in glutamate C-4 was significantly greater in Tg PD-ABAD ($58 \pm 5\%$) than in nonTg littermates (38

$\pm 7\%$; $P < 0.03$). As might be expected from the increased utilization of exogenous β -hydroxybutyrate, measurement of basal ATP levels in cerebral cortex of Tg PD-ABAD mice fasted overnight showed a statistically significant increase compared with nonTg littermates. Similarly, levels of β -hydroxybutyrate in the brains of Tg PD-ABAD mice were lower, compared with nonTg controls in view of its increased utilization in the presence of higher levels of neuronal ABAD (see animals not subjected to stroke).

 Induction of stroke in Tg PD-ABAD mice. In order to assess the possible contribution of ABAD to ischemic stress, Tg PD-ABAD and age-matched nonTg littermate control mice were subjected to a 45 min period of transient middle cerebral artery occlusion followed by a 24 hr period for evolution of the cerebral infarct. These studies utilized a protocol which we have found to provide reproducible stroke volumes and functional data (neurologic deficit scores, cerebral blood flow) (45-48). Levels of ABAD increased an additional 2-fold in Tg PD-ABAD mice after stroke, versus with uninstrumented Tg PD-ABAD mice. Compared with nonTg littermate controls, Tg PD-ABAD mice displayed strokes of smaller volume and lower neurologic deficit scores (consistent with better maintenance of neurologic function). In contrast, there was no change in cerebral blood flow comparing the Tg and nonTg animals (not shown), consistent with a direct effect of ABAD on neurons, as ABAD was overexpressed in neurons by the PDGF B chain promoter. Analysis of cerebral cortex from Tg animals showed increased ATP and decreased lactate levels compared with nonTg controls. In addition, β -hydroxybutyrate levels were lower in animals subjected to cerebral ischemia, and this finding was much more pronounced in Tg PD-ABAD mice compared with nonTg littermate controls. These data are suggested better maintenance of energy reserve and substrate metabolism in Tg PD-ABAD mice subject to ischemia. --

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Please delete the paragraph beginning on page 70, line 10 and ending on page 71, line 6 and replace with the following:

Q11 -- Increased expression of ABAD in human brain following cerebral infarction and in response to experimentally-induced cerebral ischemia [(Fig. 5)] suggests that induction of ABAD might subserve normal protective mechanisms. In view of the complexities of cellular metabolic pathways, it was necessary to prove that ABAD could promote metabolic homeostasis in response to nutritional deprivation. ABAD-transfected COS cells displayed increased energy charge and flux of acetyl-CoA through the TCA cycle in medium containing β -hydroxybutyrate compared with controls in which the active site of ABAD was mutationally inactivated. Enhanced metabolic homeostasis was reflected by maintenance of MTT reduction and morphologic phenotype in ABAD-transfected COS cells. Similarly, transgenic mice overexpressing ABAD in cortical neurons demonstrated increased flux of acetyl-CoA through the TCA cycle following β -hydroxybutyrate infusion compared with nontransgenic littermates. However, increased basal levels of ATP (and energy charge; data not shown) in brains of Tg PD-ABAD mice, even before nutritional stress, was unexpected, and suggests a more general protective potential of ABAD in response to a range of environmental challenges. This apparent increase in the overall energy charge in the presence of ABAD, implies that the enzyme may render neurons metabolically more stable and, thus, less susceptible to fluctuations in substrate availability. --

Please delete the line 31 on page 72 and replace it with the following:

Q12 -- References for Example 4 --